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# OSMOTIC CONSTITUENTS OF THE COELACANTH *LATIMERIA CHALUMNAE* SMITH

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The coelacanth *Latimeria chalumnae* occupies a unique position in the phylogenetic tree of the vertebrates as the only living representative of the crossopterygians, a group generally thought to lead from the ancestral bony fishes to the amphibians (Berg, 1958; Young, 1962; Romer, 1966). This, together with the fact that until 1939 the crossopterygians had been considered long extinct, dying out in the Cretaceous, accounts for the great interest shown in these "living fossils." However, in spite of an almost continual search since the end of the Second World War only two females and 30 males have been found to date, and there has been chemical analysis on only one specimen. On this fish, a large frozen male, Pickford and Grant (1967) analyzed the blood, Brown and Brown (1967) searched for the ornithine cycle enzymes and urea in the liver, and Cowgill, Hutchinson and Skinner (1968) looked at the mineral content of its hard and soft tissues. The offer to us by the Royal Scottish Museum of access to another specimen was therefore accepted with enthusiasm and it was thought worthwhile to confirm and perhaps extend the results on the first. As our fish was also frozen a small study was made to find the gross effect of prolonged freezing on the distribution of ions in a common fish (the freshwater perch) to help us interpret our data.

## Materials and Methods

Our specimen was a large male 32 kg weight and 1.37 m long which had been caught on March 1969 off the Grand Comore Island. It had been deep frozen and transported by air to Edinburgh. On the 5th of December (after nine months' freezing) two muscle cores and a liver core were taken from the still frozen body. On the 15th it was allowed to thaw and about 36 hours later a cast was made of the whole animal. After this the body cavity was opened and samples of body fluids and tissue taken. The fish appeared in good condition and there was no sign or smell of decay. Blood was taken from a mesentery blood vessel, the dorsal aorta, and a large dorsal blood vessel which was probably the vena cava. Tissue and body fluid samples were kept deep frozen (−20° C) until required for analysis.

The perch (*Perca fluviatilis*) used were killed by a sharp blow on the back of the head and were stored individually in sealed polythene bags at −20° C for six months. After being allowed to thaw, muscle samples were dissected from the epiaxial region and blood taken directly from the heart. The body fluid samples from both perch and *Latimeria* were centrifuged before use to remove all possible denatured protein "debris" and erythrocyte ghosts. Frozen tissues were allowed

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to thaw at room temperature and lightly blotted with filter paper; they were then weighed and the dry weight determined after heating in an oven at 105° C for 24 hours. From wet tissues 0.2 N $HNO_3$ and 1.0 N $HNO_3$ extracts were made for Na, K and Cl determination (Lutz, 1970). On the dry tissues 10% trichloroacetic acid extracts were used for Ca and Mg extraction (Lutz, 1970).

The metal ions were measured on a Unicam atomic absorption spectrophotometer SP 90 with the individual standards approximating to the ionic composition (as far as the dominant ions are concerned) of plasma and tissues extract. For Ca and Mg 0.75% EDTA was added to both samples and salines (Cook, 1969).

Chloride was measured by the electrometric method of Cotlove (1964) using a Cotlove Automatic Chloride Titrator. Osmotic pressure was measured as sample freezing point using the Ramsay-Brown apparatus (Ramsay and Brown, 1955) and by the Krogh-Baldes vapor pressure method (Krogh, 1939, page 211).

The nitrogenous compounds of muscle were estimated on tungstic acid extracts, 5 ml 10% sodium tungstate and 5 ml $\frac{2}{3}$ N sulfuric acid for each 2 g fresh muscle. For serum and bile one volume of each of the tungstic acid reagents was added to one volume of fluid diluted with 1–3 volumes distilled water. Microdiffusion methods of Conway (1962) were used for ammonium ions of body fluids and muscle extracts, for urea (urease method) and for the final determination of total non-protein nitrogen after micro-Kjeldahl digestion of samples of the filtrates. The $\alpha$-amino N of free amino acids was determined according to Frame, Russell and Wilhelmi (1943) and Russell (1944), being corrected for the color given by the ammonium ions (90% of that of $\alpha$-amino N of glycine). Trimethylamine oxide (TMAO) was estimated on tungstic acid filtrates after Kermack, Lees and Wood (1955), formaldehyde being used to hold back ammonia during the micro-diffusion of the trimethylamine. Tungstic acid filtrates were also used in the estimation of creatinine (Owen, Iggo, Scandrett and Stewart, 1954), and creatine, the latter being converted to creatinine at pH 2.2 on a boiling water bath for 2 hours.

## Results

### (a) *Effect of prolonged freezing on perch*

The results obtained from analysis of muscle and blood samples from three individuals frozen for six months are compared to the mean values for 30 normal fish (Table I). It can be seen that considerable, but uniform, changes have occurred. In blood Na has fallen to about half the *in vivo* value and shows a large rise in the frozen muscle. The fall is even greater for plasma Cl and the muscle Cl has doubled. Potassium has increased 20 fold in the post-mortem blood. Muscle potassium on the other hand has decreased steeply and the fact that it is now close to the plasma value suggests that in six months freezing K has almost equilibrated throughout the two compartments. Calcium behaves like Na with a fall in plasma and rise in muscle. A three-fold increase is seen in plasma Mg with, however, a correspondingly slight fall in muscle, leaving the ion concentration gradient between the two compartments by far the highest for this ion. It would appear that Mg is the least mobile of all the ions considered under these circumstances. No clear trend is apparent from this data for changes in tissue hydration.

To summarize, there is a general move to the equalization of concentrations

TABLE I

*Effect of prolonged freezing on the inorganic constituents of perch blood and muscle (mM/l body fluid and mM/kg tissue water)*

| | | Na | K | Ca | Mg | Cl | % water |
|---|---|---|---|---|---|---|---|
| Plasma | Normal* | 154.2 | 3.6 | 4.38 | 1.44 | 120 | — |
| | 1 | 68.9 | 60.1 | 2.90 | 4.1 | 56.1 | — |
| Serum | 2 | 71.0 | 63.5 | | 4.8 | 27.3 | — |
| | 3 | 78.8 | 63.8 | 1.50 | 4.5 | 39.2 | — |
| | Normal* | 19.9 | 143.0 | 2.6 | 15.1 | 11.6 | 80.2 |
| | 1 | 24.2 | 62.0 | 3.6 | 12.2 | 20.7 | 81.4 |
| Muscle | 2 | 30.6 | 53.8 | 2.4 | 11.5 | 16.4 | 80.1 |
| | 3 | 41.0 | 69.9 | 4.9 | 15.6 | 27.5 | 74.9 |

1, 2 and 3 refer to samples from frozen perch.
* n = 30.

during prolonged freezing, with, in six months, plasma Na and Cl falling to around half *in vivo* values and invading muscle tissue, K equilibrating throughout the body, and Ca and Mg showing similar shifts down concentration gradients. As thawing took no more than six hours at room temperature the bulk of the ion movement probably occurred in the frozen state.

(b) *Measurements from frozen* Latimeria

The results of analysis of various *Latimeria* body fluids for total osmotic pressure and osmotic constituents are seen in Table II. The actual ionic values are quite different from those reported for any living vertebrate and have obviously been influenced to an important extent by freezing. A wide variation is seen for most parameters and the two most important factors causing this would be unequal ion migration during post-mortem processes and the possible dilution of some body fluids by ice crystals formed during the prolonged freezing of the tissues. The latter phenomenon would primarily affect the osmotic pressure and total ion concentration in the fluids while increasing slightly the osmotic concentration in parts of muscle and other tissues. In the blood and coelomic fluid the total ion content varies directly with the osmotic pressure and, since in fishes the initial *in vivo* values for these fluids are similar (Lutz, 1970), the large variance found for frozen *Latimeria* most likely reflects dilution. If this is so then the higher values are probably the most reliable *i.e.*, those values around 1000 milliosmoles. In perch the bile has an osmotic pressure similar to that of plasma (Lutz, 1970) and if this is generally true of fishes then the results found here would further support the suggestion that the lower osmotic and total ion values in Table II result from ice crystal formation. It seems probable then that the vena cava samples are further complicated by dilution factors. Comparing with the values from frozen perch it is seen that in our *Latimeria* blood there is a higher Na and Cl, similar K and similar Ca.

The nitrogenous constituents of *Latimeria* body fluids show that the vena cava has also the lowest value of urea. Urea is clearly an important constituent with

TABLE II

*Osmotic constituents of* Latimeria *body fluids*

| | Dorsal aorta serum | Vena cava serum | Mesentery vessel serum | Coelomic fluid | Bile |
|---|---|---|---|---|---|
| Na | 88.6 | 89.5 | 135.4 | 101.4 | 73.8 |
| K | 62.5 | 35.6 | 60.2 | 66.5 | 58.2 |
| Ca | 1.74 | 1.00 | 2.30 | 2.9 | 1.75 |
| Mg | 3.81 | 1.56 | 9.44 | 11.7 | 95.8 |
| Cl | 86.53 | 88.44 | 125.4 | 105.4 | 77.5 |
| Urea | 337 | 242 | | | 289 |
| Trimethylamine oxide | | 109.4 | | | 107.0 |
| Total NPN mg-atoms/l | | 786 | | | 1224 |
| Total inorganic ions | 243.2 | 216.1 | 332.7 | 287.9 | 215.3 |
| Osmotic pressure milliosmoles | 968 | 766 | 1138 | | 1135 |

values ranging from 240–340 mM/l. Trimethylamine oxide is also present in large amounts with concentrations just less than half those of urea and accounting from some 10–15% of the total non-protein nitrogen found. Free trimethylamine was not detected in plasma samples indicating that decomposition had not occurred to any significant extent.

Table III shows the osmotic composition of muscle and liver samples. As with blood the ion results are quite uncharacteristic of vertebrate tissue indicating considerable post-mortem changes. The consistency of the results as shown by the standard errors is, however, much better.

The liver differs from muscle principally in the very low water content (due to an extraordinary high fat content) and in the much higher values for Na and Cl.

TABLE III

*Osmotic constituents of* Latimeria *muscle and liver (mM/kg tissue water)*

| | Muscle | | Liver | |
|---|---|---|---|---|
| | Mean | Number | Mean | Number |
| Na | 30.63 ± 2.294 | 8 | 98.53 ± 4.673 | 3 |
| K | 73.50 ± 8.365 | 6 | 50.10 ± 11.67 | 3 |
| Ca | 1.77 ± 0.253 | 6 | 1.94 ± 0.358 | 3 |
| Mg | 14.36 ± 1.143 | 7 | 9.53 ± 0.632 | 3 |
| Cl | 35.32 ± 4.73 | 5 | 115.5 ± 2.201 | 3 |
| Urea | 421.5 [378–465] | 2 | | |
| Trimethylamine oxide | 290 | 1 | | |
| Creatine | 31.9 [25.6–38.3] | 2 | | |
| Creatinine | 1.85 [1.5–2.2] | 2 | | |
| Amino acids | 60.35 [50.7–70.0] | 2 | | |
| Total NPN mg-atoms/kg water | 1423 [1192–1654] | 2 | | |
| % water | 73.66 ± 1.51 | 5 | 48.94 ± 2.21 | 3 |

[ ] = range.
± = standard error.

High concentrations of these two ions are characteristic of the livers of a variety of vertebrates including mackerel (Becker, Bird, Kelly, Schilling, Soloman and Yound, 1958), perch (Lutz, 1970) and the rat (Widdowson and Dickerson, 1964), and this has been interpreted as indicating a common pattern of high intracellular values of Na and Cl for this tissue (Lutz, 1971). It seems likely that this generalization can be extended to the coelacanths.

The nitrogenous constituents further illustrate the importance of urea and trimethylamine oxide, both being found in muscle in significantly higher concentrations than in the body fluids. As in all vertebrates creatine and creatinine were found, the former presumably present partly as creatine phosphate *in vivo*. No free trimethylamine was detected.

## Discussion

A comparison of these results with those from the only other *Latimeria* examined so far is of interest (Table IV). Considering their history, data from both specimens agree quite well in values for $K_p$ (plasma K) and $K_m$ (muscle K), $urea_p$, $mOsm_p$, $Na_m$ and $Mg_m$; and this together with the very high $Mg_p$ found by Pickford and Grant (1967) suggests that considerable post-mortem ion shifts had also occurred in the first specimen. The results disagree strikingly in $Na_p$ and $Cl_p$, with Pickford and Grant's specimen having almost double the values that we find. Although trimethylamine oxide was not looked for in the first *Latimeria*, the results of Pickford and Grant (1967) appear to exclude its presence in significant amounts, finding as they report, that urea made up 88% of the total blood NPN. The method of analysis used in this study is however quite specific and we have

Table IV

*A comparison of the data of the blood and muscle of two frozen specimens of* Latimeria (*mM/l blood, mM/l tissue water*)

| Constituent | Blood serum | | Muscle | |
|---|---|---|---|---|
| | 1* | 2ˣ | 1 | 2⁺ |
| Na | 104.5 | 181 | 30.6 | 29.3 |
| K | 52.8 | 51.3 | 73.5 | 106.6 |
| Ca | 1.68 | 3.5 | 1.8 | 2.7 |
| Mg | 4.94 | 14.4 | 14.4 | 10.0 |
| Cl | 100.1 | 199.0 | 35.3 | 0.4 |
| Urea | 290 | 355 | 422 | |
| Trimethylamine oxide | 109.4 | | 290 | |
| Total NPN | 786 | 959 | 1423 | |
| Milliosmoles | 957 | 1181 | | |

* Arithmetical means from Table II.

ˣ Pickford and Grant (1967).

⁺ Cowgill, Hutchinson and Skinner (1968).

The TMAO and total NPN values of 1* serum are single values for vena cava blood which is apparently diluted. Calculations from the urea concentrations of vena cava and dorsal aorta sera suggest TMAO value of 152 mM and a NPN of 1095 mg-atoms for the dorsal aorta blood, which are probably closer to the *in vivo* values.

confidence in these results. The very low values reported by Cowgill *et al.* (1968) for Cl are extraordinary and must be regarded as spurious, since they would indicate that this animal had a negligible to zero extracellular space in its muscle tissue. Perhaps the method used of x-ray fluorescence is not particularly applicable to chloride analysis in these systems.

The range of osmotic pressure values found for our more reliable samples is 968–1138 milliosmoles, similar to that judged best by Pickford and Grant (1181 mOsm). It is probable that these cover the *in vivo* value for the animal and point to it being in approximate osmotic equilibrium with sea water. Whether it is slightly hyper-osmotic (like the sharks) or hypo-osmotic is not possible to say. Pickford and Grant give a value of 1090 milliosmoles (derived from salinity determinations of Dr. M. S. Gordon) for sea water in the region of Grand Comore Island.

The amounts of urea and trimethylamine oxide found in blood and muscle are considerable, and the muscle values as far as we can find, are by far the highest yet recorded for any animal. This may be of some significance as post-mortem changes are most likely to be accompanied by a decrease in both constituents.

As far as the inorganic ions are concerned similar post-mortem changes to those found in perch undoubtedly have occurred as they would account for the very high $K_p$ and $Mg_p$ found in *Latimeria* blood and for the fact that $K_p$ approaches $K_m$.

The very low $Na_p$ and $Cl_p$ found by us would also agree with this suggestion, and if true would mean that the $Na_m$ and $Cl_m$ are *in vivo* substantially less than the values shown here.

It would appear that the coelacanth is similar to the elasmobranchs and teleosts in having internal salt concentrations much less than that of the surrounding sea water ($\frac{1}{3}$–$\frac{1}{2}$ S.W.). Like the elasmobranchs, and in contrast to the teleosts, they are also in approximate osmotic balance with their environment, the difference in salt concentration being made up in both cases by the nitrogenous compounds urea and TMAO. It seems that teleosts are ureogenic, having the full complements of ornithine-urea cycle enzymes (Huggins, Skutsch and Baldwin, 1969) although this had previously been denied (Brown and Cohen, 1960). Their ancestors were probably also ureogenic.

The first crossopterygians and dipnoans were probably ureogenic as are their present-day descendants including *Latimeria* (Brown and Brown, 1967) and *Protopterus* (Janssens and Cohen, 1966). While most crossopterygians remained in fresh water, one line leading to the amphibians and hence all higher vertebrates, a side-branch, the coelacanths, invaded sea water in the Triassic (Romer, 1966). It is suggested that this group became adapted to the marine environment by the same basic methods as the elasmobranches had evolved in the Silurian, *i.e.,* by developing a physiological tolerance to high urea and TMAO concentrations, and evolving mechanisms for the active retention of both components. Both groups are therefore ureosmotic.

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## Summary

1. Samples of blood, bile, muscle and liver from a frozen specimen of the coelacanth *Latimeria chalumnae* were analyzed for ions and nonprotein nitrogenous compounds.

2. Mean values (mM/l) for blood serum (hemolyzed) and bile (figures in brackets) were Na 104.5 (73.8) K 52.8 (58.2), Ca 1.68 (1.75), Mg 4.49 (4.0), Cl 100.1 (77.5), urea 290 (289), trimethylamine oxide 109.4 (107.0), total NPN 786 mg-atoms (1224), osmolality 957 milliosmoles (1135).

3. Mean values (mM/kg water) for muscle and liver (figures in brackets) include Na 30.6 (98.5), K 73.5 (50.1), Ca 1.8 (1.9), Mg 14.3 (9.5), Cl 35.3 (115.5), urea 422, trimethylamine oxide 290, total NPN 1423.

4. A study of the effect of prolonged freezing on the electrolyte distribution of the perch *Perca fluviatillis* was made and compared with the above results.

5. *Latimeria* differs from a marine teleost and resembles an elasmobranch in having large quantities of urea and trimethylamine oxide in both blood and muscle, and a high osmotic concentration near that of sea water.

## LITERATURE CITED

Becker, E. L., R. Bird, J. W. Kelly, J. Schilling, S. Soloman and N. Yound, 1958. Physiology of marine teleosts—1. Ionic composition of tissues. *Physiol. Zool.*, **31**: 224–227.

Berg, L. S., 1958. *System der Rezenten und Fossilen Fischartigen und Fische.* V. E. B. Deutscher Verlag der Wissenschaften, Berlin, 311 pp.

Brown, G. W., Jr., and S. G. Brown, 1967. Urea and its formation in Coelacanth liver. *Science*, **155**: 570–573.

Brown, G. W., Jr., and P. P. Cohen, 1960. Activities of urea-cycle enzymes in various higher and lower vertebrates. *Biochem. J.*, **75**: 82–91.

Conway, E. J., 1962. *Microdiffusion Analysis and Volumetric Error.* [5th ed.] Crosby Lockwood, London, 467 pp.

Cook, P., 1969. *Atomic Absorption spectrophotometry.* [2nd ed.] Pye Unicam Ltd., Cambridge, England, 44 pp.

Cotlove, E., 1964. Determination of chloride in biological materials. Pages 277–392 in D. Glick, Ed., *Methods of Biochemical Analysis. XII.* John Wiley and Sons, New York.

Cowgill, U. M., G. E. Hutchinson and H. C. W. Skinner, 1968. The elementary composition of *Latimeria chalumnae* Smith. *Proc. Nat. Acad. Sci. U. S. A.*, **60**: 456–463.

Frame, E. G., J. A. Russell and A. E. Wilhelmi, 1943. The colorimetric estimation of amino nitrogen in blood. *J. Biol. Chem.*, **149**: 255–270.

Huggins, A K., G. Skutsch and E. Baldwin, 1969. Ornithine-urea cycle enzymes in teleostean fish. *Comp. Biochem. Physiol.*, **28**: 587–602.

Janssens, P. A., and P. P. Cohen, 1966. Ornithine-urea cycle enzymes in the African lungfish *Protopterus aethiopicus. Science*, **152**: 358–359.

Kermack, W. O., H. Lees and J. D. Wood, 1955. Some non-protein constituents of the tissues of the lobster. *Biochem. J.*, **60**: 424–428.

Krogh, A., 1939. *Osmotic Regulation in Aquatic Animals.* University Press, Cambridge, England, 242 pp.

Lutz, P. L., 1970. Osmotic and Ionic Regulation in the perch, *Perca fluviatilis. Ph.D. thesis, University of Glasgow,* 116 pp.

Lutz, P. L., 1971. Extracellular spaces and composition of various tissues of perch. *Comp. Biochem. Physiol.,* in press.

Owen, J. A., B. Iggo, F. J. Scandrett and C. P. Stewart, 1954. The determination of creatinine in plasma or serum, and in urine; a critical examination. *Bioch. J.*, **58**: 426–437.

Pickford, G. E., and F. B. Grant, 1967. Serum osmolarity in the coelacanth *Latimeria chalumnae;* urea retention and ion regulation. *Science,* **155**: 568–570.

Ramsay, J. A., and R. H. J. Brown, 1955. Simplified apparatus and procedure for freezing point determinations upon small volumes of fluid. *J. Scient. Instrum.*, **32**: 373–375.

Romer, A. S., 1966. *Vertebrate Paleontology.* [3rd ed.] University of Chicago Press, Chicago, 468 pp.

Russell, J. A., 1944. Note on the colorimetric determination of amino nitrogen. *J. Biol. Chem.*, **156**: 467–468.

Widdowson, E. M., and J. W. T. Dickerson, 1964. Chemical Composition of the Body. Pages 2–248 in C. L. Conar and F. Bromer, Eds., *Mineral Metabolism 11A,* Academic Press, New York and London.

Young, J. Z., 1962. *The Life of Vertebrates.* [2nd ed.] Oxford University Press, London, 820 pp.